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Protection against murine osteoarthritis by inhibition of the 26S proteasome and lysine-48 linked ubiquitination

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ABSTRACT

Objectives: To determine whether the process of ubiquitination and/or activity of the 26S proteasome are involved in the induction of osteoarthritis (OA).

Methods: Bovine cartilage resorption assays, chondrocyte cell-line SW1353 and primary human articular chondrocytes were used with the general proteasome inhibitor MG132 or vehicle to identify a role of the ubiquitin–proteasome system (UPS) in cartilage destruction and matrix metalloproteinase-13 (MMP13) expression. In vivo, MG132 or vehicle, were delivered subcutaneously to mice following destabilisation of the medial meniscus (DMM) induced OA. Subsequently, DMM was induced in Lys-to-Arg (K48R and K63R) mutant ubiquitin (Ub) transgenic mice. Cytokine-signalling in SW1353s was monitored by immunoblotting and novel ubiquitinated substrates identified using Tandem Ubiquitin Binding Entities purification followed by mass spectrometry. The ubiquitination of TRAFD1 was assessed via immunoprecipitation and immunoblotting and its role in cytokine signal-transduction determined using RNA interference and real-time RT-PCR for MMP13 and interleukin-6 (IL6).

Results: Supplementation with the proteasome inhibitor MG132 protected cartilage from both cytokine-mediated resorption and degradation in vivo in mice following DMM-induced OA. Using transgenic animals only K48R-mutated Ub partially protected against OA compared to wild-type or wild-type Ub transgenic mice and this was only evident on the medial femoral condyle. After confirming ubiquitination was vital for NF-κB signalling and MMP13 expression, a screen for novel ubiquitinated substrates involved in cytokine-signalling identified TRAFD1; the depletion of which reduced inflammatory mediator-induced MMP13 and IL6 expression.

Conclusions: Our data for the first time identifies a role for ubiquitination and the proteasome in the induction of OA via regulation of inflammatory mediator-induced MMP13 expression. These data open avenues of research to determine whether the proteasome, or K48-linked ubiquitination, are potential therapeutic targets in OA.

Keywords

Ubiquitin, proteasome, osteoarthritis, animal model, MMP13

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INTRODUCTION

Osteoarthritis (OA) is the most prevalent joint disease,[1] the typifying feature of which is the progressive degradation of articular cartilage.[2] Cartilage itself is composed of an extracellular matrix (ECM) rich in proteoglycan (principally aggrecan) and collagen (mainly type II) maintained by the sole cell type, the chondrocyte. Remodelling and turnover of this ECM in normal physiology is mediated through the regulation of the expression of matrix components and matrix-degrading enzymes, the metalloproteinases.[3] The phenotype of the chondrocytes shifts in OA, through as yet unidentified processes, to favour catabolism with an increase in metalloproteinase expression evident. Matrix metalloproteinase (MMP)-13 is the generally accepted type II collagen-degrading proteinase in OA with pathological aggrecan cleavage mediated by ADAMTS enzymes, most probably ADAMTS-5.[4] Mechanisms to alter the expression of these enzymes in OA are potential therapeutic targets in what is currently a disease only treatable by joint replacement surgery.

Cellular proteins exist in a dynamic state with multiple pathways leading to their degradation, the best characterised being the ubiquitin–proteasome system (UPS). The UPS plays a pivotal role in the degradation of proteins important in numerous cellular processes including signal transduction pathways, a notable example being that of the NF-κB pathway via the regulation of IκB degradation. IκBs normally bind and sequester NF-κB dimers in the cytoplasm, but following their degradation the NF-κB complex translocates to the nucleus and regulates inflammatory signalling. Like most proteins destined for degradation via the UPS, IκB is covalently modified by the attachment of the small polypeptide ubiquitin (Ub). Subsequently, additional Ub polypeptides can be covalently attached to the first Ub to create a poly-Ub chain that eventually directs the client protein for proteasomal degradation.[5, 6]

Ub itself is a highly-conserved 76 amino acid polypeptide expressed by all eukaryotes. The covalent attachment of Ub to cellular proteins, ubiquitination, occurs at lysine (K) residues. Ub itself contains seven K residues which allows for different poly-Ub chains to be generated including K48 chains which target proteins to the proteasome. K63 and linear Ub

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chains have emerged as having important roles in signal transduction pathways, the most studied again being the NF-κB pathway.[6]

The aim of this study was to investigate the role of Ub and the UPS in the induction of OA in a murine model and to identify novel targets of Ub, all of which may have potential therapeutic relevance.

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METHODS

Cells, samples and treatments

This study was performed with Ethical Committee approval from the Newcastle and North Tyneside Health Authority, UK. Human articular chondrocytes (HACs) were from consenting patients undergoing knee joint replacement surgery and were isolated and cultured as described.[7] Human chondrosarcoma cells (SW1353) were cultured as described.[8] Cells were seeded one day before treatment and cultured in serum-free medium overnight prior to stimulation. Cells were stimulated with human recombinant IL-1α (0.5 ng/ml) or 0.5 μg/ml poly(I-C) (Invivogen, Source BioScience LifeSciences, Nottingham, UK). Where indicated, cells were treated 1 h before and during stimulation with MG132 (5 μg/ml) (Tocris biosciences, R&D Europe Ltd. Abingdon, UK or Sigma-Aldrich Ltd. Poole, UK).

Gene depletion/RNA intereference (RNAi)

Cells were transfected with siRNA (50 nM) for 48 h using Dharmafect 1 (Dharmacon, Thermo Fisher Scientific, Epsom, UK) according to the manufacturer's protocol and as described previously.[7, 8] The following siRNAs were used: siGENOME SMARTpool: siGenome TRAFD1 siRNA (M-004097-01-0005) and siGenome Non-targeting siRNA pool #2 (D-001206-14-20)(siCon)(Dharmacon).

Immunoblotting

Whole cell lysates were prepared as described but supplemented with 20 mM N-ethylmaleimide, to block deubiquitination.[7] Protein extracts were separated by SDS-PAGE, transferred to PVDF or nitrocellulose membranes and probed with the following antibodies: anti-IκBα; anti-phospho-NF-κB p65; anti-FLAG (Cell Signaling, New England Biolabs (UK) Ltd, Hitchin, UK); anti-GAPDH (Chemicon, Millipore (U.K.) Ltd., Watford, UK); anti-TRAFD1 (Santa Cruz Biotechnology (SCBT), Inc., Insight Biotechnology Ltd., Wembley, UK), and anti-Ubiquitin (Dako UK Ltd, Ely, UK).

Enrichment of proteins with Tandem Ubiquitin Binding Entities (TUBES)

SW1353 protein extracts were prepared following 30 min stimulation with IL-1, 1 h stimulation with poly(I-C), or under basal conditions, all in presence of MG132. Cell lysis

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buffer (50 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP40, 10% glycerol) was supplemented with protease inhibitor cocktail (Sigma-Aldrich) and 20 mM N-ethylmaleimide, and concentrated with a 9 kDa cut-off filter (Pierce, Thermo Fisher Scientific). 4.5 mg of protein were pre-cleared with 50 µl 50% slurry of control agarose for 30 min, and then incubated with 200 µl of 50% slurry TUBE 2 (tebu-bio Ltd, Peterborough, UK) overnight at 4^{o}C.[9] Enriched proteins were washed thrice with TBS-T (20 mM Tris-HCl pH 8.0, 0.15-0.2 M NaCl, 0.1% Tween-20), eluted with 200 µl 0.2M glycine-HCl pH 2.5 and concentrated with a 10 kDa cut-off filter (Amicon, Millipore, Thermo Fisher Scientific, Epsom, UK). Enrichment of ubiquitinated polypeptides was visualised by immunoblotting prior to being reduced with 1 mM DTT, alkylated with 5 mM chloroacetamide, and trypsin (Promega, Southampton, UK) digested at 37^{o}C overnight before identification by mass spectrometry (MS).

Mass spectrometric analysis and data analysis

MS was performed by Dundee Cell Products Ltd. (Dundee, UK) on an UltiMate™ 3000 nLC (Thermo scientific) coupled to a LTQ Orbitrap XL (Thermo Scientific). RAW data files were extracted with Raw2MSM and searched using Mascot Search Engine. A maximum of 2 missing tryptic cleavages and the following modifications were selected for database search criteria: fixed modifications: Carbamidomethyl (C); variable modifications: Acetyl (N-term), Dioxidation (M), Gln->pyro-Glu (N-term Q), Oxidation (M), with/without GlyGly (K). The relative quantification of proteins (spectral count) was performed using an exponentially modified abundance index (emPAI) as described.[10]

TRAFD1 cloning and immunoprecipitation

A human TRAFD1 ORF clone, IRQMp5018H057D (Source Bioscience), was PCR amplified using primers 5'-CAGTGTGGTGGAATTTCGTCCCTGGAAGAGCTAAA-3' and 5'-GATATCTGCAGAATTCTCCTCCTCTTCCTCTTCTGC -3' and subcloned into a modified pcDNA4 (Life Technologies, Paisley, UK) vector using In-Fusion (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) to create a C-terminal FLAG-tag fusion protein. SW1353s were transfected using JetPEI (Source Bioscience) and TRAFD1 expression detected by immunoblotting. Transfected cells were treated as indicated. Over-expressed TRAFD1 was immunoprecipitated from 150 µg of whole-cell-lysate with 1.5 µg of anti-FLAG antibody

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(Sigma-Aldrich) overnight at 4°C, then incubated with 20 µl protein G PLUS-agarose (SCBT) for 1 h at 4°C. Bound proteins were washed once with lysis buffer and twice with TBS-T and eluted with 50 µl 2 x Laemmli sample buffer prior to SDS-PAGE and immunoblotting with the anti-FLAG or anti-Ub (after autoclaving of membranes) antibodies.

Real-time reverse transcription-PCR

Total RNA from cells was extracted using Cells-to-cDNA II kit, RNAse-free DNAse (Life Technologies) treated, and cDNA synthesised as described.[11] Primers and probes were as published [7] or designed by the Universal Probe Library (Roche Applied Science, Burgess Hill, UK) and were: *TRAFD1* F 5'-CAGCCTCAAGAGACCTCACC-3' and R 5'-TCCAGGTAACCAGAAGACAGGT-3' with probe#44; *IL6* 5'-GATGAGTACAAAAGTCCTGATCCA-3' and R 5'-CTGCAGCCACTGGTTCTGT-3' with probe#40. Real-time RT-PCR performed and analysed as described.[12]

***In vitro* cartilage degradation assay**

Cultured bovine cartilage discs were incubated with serum-free DMEM containing IL-1 (1 ng/ml) and Oncostatin M (OSM) (10 ng/ml) ± DMSO/or MG132 (5 µg/ml) and refreshed on day 7. Residual cartilage explants were papain digested overnight at 65°C. Hydroxyproline (OHPro) and glycosaminoglycan (GAG) release were assayed as described.[13] Conditioned media was assessed at day 7 and 14 (presented) for damaged cells using the ToxiLight™ BioAssay Kit (Lonza, Slough, UK). Day 0 cartilage was freeze-thawed thrice as a positive control of cell damage.

Experimental osteoarthritis

Experiments were performed on wild-type C57BL/6J or mutant-Ub transgenic mice housed in standard cages with *ad libitum* food and water in accordance with UK Home Office regulations. OA was induced in 8 week old animals, following surgical sectioning of the medial meniscotibial ligament with sham operation as control (data not shown).[14] MG132 or vehicle (50% DMSO/50% polyethylene glycol 300) was delivered via osmotic mini-pump (1004; ALZET Osmotic Pumps, Cupertino, CA, USA) implanted subcutaneously and delivering 0.125 µl/h. Based upon previous studies (e.g. [15]), a single concentration of MG132 (6.6

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mg/ml) was selected which delivered 1 mg/kg/day (8 mice/group). Osmotic pumps were replaced after 4 weeks. 8 weeks post-surgery mice were euthanized and knee joints harvested for histological examination and scoring. On average 14 sections/joint, harvested at approximately 80 μm intervals, were used for histological scoring by two blinded scorers.[16, 17] Averaged scores for the maximally affected sections are presented. Transgenic mutant-Ub animals were as described,[18, 19] where all three transgenic mouse lines express human Ub as a linear fusion with a N-terminal 6 x His tag and a C-terminal enhanced green fluorescent protein (eGFP), the latter being cleaved to generate eGFP and His-tagged Ub. Since all Ub-transgenes have an N-terminal His-tag none can support linear poly-ubiquitination. K48R Ub and K63R expressing mice also have impaired K48 and K63 poly-ubiquitination respectively. Genotyping of mice was via GFP visualisation [19] and PCR using Ub forward primer: 5'-CATGCAGATCTTCGTGAAGACCCTGACCGGCAAG-3' and GFP reverse primer: 5' CCTGCCGGTGGTGCAGAT-3' followed by restriction digestion with the endonuclease NgoMIV which only cleaves the K48R PCR amplicon.

Statistical analysis

All values are given as mean values of replicates with error bars representing the standard error of the mean (SEM). Mann-Whitney U-test was used to assess independent pairs with a Student's t-test used for real-time PCR data analysis using Graphpad Prism Software (GraphPad Software, Inc., La Jolla, USA), where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

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RESULTS

Inhibition of the proteasome reduces cartilage destruction and *MMP13* expression

MG132 reduces the degradation of ubiquitin-conjugated proteins by the 26S proteasome complex and because this diverts ubiquitin pools into futile conjugates it has the effect of disrupting Ub homeostasis. Since poly-ubiquitination is strongly associated with NF-κB signal transduction,[20] we evaluated the effect of MG132 on cytokine-induced cartilage destruction using an established bovine cartilage model.[13] MG132 completely ameliorated the cartilage damage, as measured using glycosaminoglycan (GAG) and hydroxyproline (OHPro) release as surrogates for aggrecan and collagen destruction, mediated by the IL-1+OSM combination (Fig. 1A and B). Potential cytotoxicity of the MG132 treatment was assessed at day 7 and 14 (presented Fig. 1C) and showed low toxicity compared to freeze-thawed cartilage or the IL-1+OSM cytokine combination.

Cartilage-collagen turnover is mediated by MMPs, with MMP13 the accepted collagenase in OA. In both HAC and SW1353 cells, MG132 completely abolished the IL-1 or poly(I-C) (pIC) induced *MMP13* expression, the latter stimulus being a double-stranded RNA mimic which is a potent inducer of *MMP1*3 in HAC (Fig 1D).[7] Since IL-1 and poly(I-C) activate the NF-κB signalling pathway, we examined the effect of MG132 on the activation of this pathway by assessing the phosphorylation status of the NF-κB component p65 and the degradation of IκBα, which is a consequence of K48 poly-ubiquitination. Interestingly, MG132 caused an increase in P-p65 even under basal conditions, as has previously been reported.[21] Using an early time-point of stimulation (15 min), at which IκBα degradation is initiating,[7, 8] we observed MG132 caused an increase in higher molecular weight IκBα immune-reactive products which we ascribed to be K48 poly-ubquitinated forms (Fig 1E).

Inhibition of the proteasome reduces induced-OA in mice

Given the ability of MG132 to protect against cytokine-induced cartilage destruction, we tested the efficacy of the compound in the murine DMM model of post-traumatic OA. MG132 was delivered systemically post-surgery for the eight week duration of the experiment at which point OA was assessed by histological scoring.[16] By this time-point in the model, cartilage lesions develop primarily on the central weight-bearing region of the

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medial-tibial plateau and to a lesser extent the medial-femoral condyles.[14] MG132 significantly reduced cartilage damage in the most affected medial-tibial plateau but showed little evidence of efficacy for the less damaged femoral condyle (Fig. 2A and B). Summation of the medial-femoral and tibial histological scores demonstrated an overall protection of cartilage loss in MG132 administered animals (Fig. 2B). Similarly to other studies (e.g. [22]), the administration of MG132 at this dose and for this length of time had no effect on the activity/wellbeing of the mice and had no impact on the weight of the animals.

K48 poly-ubiquitination plays a role in DMM-induced OA

To determine the mechanism by which MG132 protected the mice from induced-OA, we performed DMM surgery on transgenic mice expressing one of three mutated forms of Ub, unable to form specific poly-Ub chains, in addition to their endogenous Ub genes.[18] When examining the medial tibial plateau none of the mutant-Ub mice were protected from OA with in fact a significant enhancement of the OA score of the K63R mice (Fig. 3A and B). However, the medial femoral condyles of the K48R mice showed a significant reduction in the OA score compared to either 'wild-type' animals or 'wild-type' Ub transgenic animals (Fig. 3A and B). Overall summation of the histological scores from both medial plateaus revealed no significant differences between transgenic and 'wild-type' animals (Fig. 3B).

TRAFD1 positively regulates induced MMP13 expression

Many components of signalling pathways are known to be modulated by Ub conjugation. Therefore we attempted to identify novel proteins ubiquitinated under either basal or stimulated conditions which may be important OA given our mouse-model findings, using chondrosarcoma SW1353 cell extracts. Ubiquitinated proteins were first enriched using TUBEs which have a nanomolar binding affinity for poly-ubiquitinated proteins.[9] After confirmation of the enrichment of Ub conjugated proteins (Fig. 4A, mid-panel), proteins were identified by MS using spectral counts to quantify relative abundance. We identified 176 proteins in total and the protein with the highest emPAI (exponentially modified protein abundance index) was ubiquitin in all samples (Supplementary table). Identified proteins with a reported role in NF-κB signalling are listed (Table 1). Of these, TRAFD1 was identified only under basal conditions and has been identified as a negative regulator of cytokine

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signalling.[23] We therefore confirmed the association of TRAFD1 with ubiquitin by over-expression and immunoprecipitation of the protein followed by immunoblotting with anti-Ub antibody (Fig. 4B). Using RNAi we specifically depleted TRAFD1 from SW1353 cells (Fig. 4C) and measured IL-1- or poly(I-C)-induced gene expression. Depletion of TRAFD1 reduced both basal and induced expression of *MMP13* (Fig. 4D). Previous work with TRAFD1 had focussed on *IL6* regulation, again similar to our findings with *MMP13*, we observed a reduction in induced *IL6* expression following TRAFD1 depletion (Fig. 4E).

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DISCUSSION

To our knowledge this is the first detailed study to evaluate the therapeutic potential of inhibition of the UPS system for OA.

MG132 is a peptide aldehyde which directly inhibits the active site within the 20S core particle, the catalytic sub-complex of the 26S proteasome. Using the DMM OA model we observed that 8-weeks post surgery, MG132-administered mice had a significant protection against OA-like cartilage erosions. Similarly, using a well established bovine *ex vivo* model of cartilage destruction, mediated by pro-inflammatory cytokines,[13] MG132 totally blocked resorption. Proteasomal inhibitors are considered as potential remedies for autoimmune and inflammatory diseases, including rheumatoid arthritis (RA) and have shown efficacy in animal models, including in rat models of OA.[15, 24, 25] This is principally via their ability to inhibit NF-κB activation.[5] Thus, it was perhaps not surprising that here MG132 prevented cartilage destruction in our explant model, presumably via limiting IκBα degradation, NF-κB signal transduction and *MMP13* expression, all of which we demonstrated *in vitro*.

The NF-κB pathway inhibitor IκB was the first identified substrate of the UPS,[26] where following its K48 poly-ubiquitination it is degraded by the proteasome. In fact, multiple different Ub-conjugated proteins are involved in NF-κB signalling. For example, linear poly-ubiquitination of NEMO, K63 poly-ubiquitination of TRAF6 (itself the responsible E3 ligase), and K11 poly-ubiquitination of RIP1, all regulate the NF-κB pathway.[20] Similarly, deubiquitination plays an important role in NF-κB signalling, exemplified by A20.[27]

Although MG132 is a proteasome inhibitor it disrupts the entire UPS, leading to an accumulation of K48 poly-ubiquitination protein and a lack of bioavailable mono-Ub. In an attempt to resolve the mode of action of MG132 in the protection of DMM-induced OA we used 'wild-type', K48R and K63R Ub transgenic mice in further DMM studies. We observed that the K63R mice developed modestly, but significantly, worse OA. K63 poly-Ub chains are involved in range of cellular processes including intracellular trafficking and autophagy where their formation causes proteins to accumulate to the aggresome.[28] Autophagy is protective for healthy cartilage, where loss of the process is associated with cell death and OA-onset.[29] We therefore speculate that the K63R mice would have reduced autophagy, explaining the observed increase in OA score. Our data also indicates that linear poly-

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ubiquitination has little importance in DMM-induced OA since all three transgenic Ub mice are incapable of this form of conjugation with their respective Ub-transgene. Somewhat pheno-copying the MG132 data, K48R transgenic animals showed significant protection from DMM-OA, but only on the less affected medial-femoral condyle, suggestive of a delay in OA-onset. These data warrant further investigation into the role of K48 poly-ubiquitination at earlier time points during DMM where lesions on the tibial plateau are initiating. A limitation of these studies is that all the transgenic mice are still able to make all poly-Ub conjugates because they still express the four copies of the endogenous Ub genes, UBA52, RPS27A, UBB and UBC,[30] although data suggest the transgenic mutant Ub acts as a dominant negative form.[18] A further limitation is that the transgenic animals were maintained on FVB/N genetic background, rather than C57Bl/6, although we still observed reduced OA in K48R mice compared to the WT-Ub transgenics or 'WT' C57Bl/6 mice, which themselves developed OA indistinguishable from each other (Fig 3).

Given the protection mediated by both MG132 and the K48R Ub transgene the data indicate a role for the 26S proteasome and potentially NF-κB signalling in DMM-OA. NF-κB signalling is the major inflammatory pathway but has yet to be investigated in DMM-induced OA, although the pathway has been extensively studied in chondrocytes and its inhibition can suppress OA in an experimental model.[31] In fact the role of inflammation, and in particular IL-1, in OA remains controversial.[32] Importantly, although we used pro-inflammatory cytokines to study NF-κB activation and *MMP13* expression, inflammatory signalling pathways, including NF-κB, can be activated by other signals including mechano-transduction.[33] Chondrocytes are well known to respond to biomechanical signals to maintain cartilage homeostasis. The magnitude of these signals are critical for the control of the activation or inhibition of pro-inflammatory genes, even in the absence of classical inflammatory stimuli. Further, DMM-induced OA is mechanosensitive with several pro-inflammatory genes that activate NF-κB, including *IL1b* and *IL6*, induced early following surgery and suppressed by joint immobilisation [34] - adding validity to our use of cytokines *in vitro* and our emphasis on NF-κB signalling. However, a number of other signalling pathways important for cartilage development and integrity are regulated by the UPS including TGF-β and Wnt signalling.[35, 36] Our data cannot preclude that MG132 or the

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K48R Ub-transgene may be affecting these or other pathways to reduce the cartilage damage induced following DMM surgery.

Given that multiple signalling pathways converge on the upregulation of aggrecanases and collagenases, especially *MMP13*, we sought to identify potentially novel Ub involved in such pathways using a novel proteomic method. From these proteomic data we identified TRAFD1, although we were unable to verify that the protein was directly ubiquitinated since the identified peptide lacked a characteristic lysine-diglycine modification found after trypsin digestion of ubiquitinated peptides. However, TRAFD1 has been previously identified as being ubiquitinated in large-scale proteomic screens for Ub-modified proteins,[37-39] with K103, K129 and K485 of the human protein showing modification. Here, we overexpressed and immunoprecipitated a tagged version of TRADF1 and confirmed the co-immunoprecipitation of Ub, presumably conjugated to TRAFD1.

We chose TRAFD1, also known as FLN29, for further study based upon previous work linking the protein to toll-like receptor (TLR) and retinoic acid-inducible gene 1 (RIG-1)-like helicase (RLH) signalling,[23, 40] and thus NF-κB signalling. In these studies, contrary to our findings, TRAFD1 is reportedly a negative regulator of TLR and RLH signalling.[23] The discrepancies between our findings and those published could perhaps be explained by differences in the cells or species used but clearly warrant further investigation. TRAFD1 was also reportedly important for IRF-3 dimerisation,[23] a process important for poly(I-C)-induced *MMP13* expression by chondrocytes.[8]

Finally, Ub is a member of a family of ubiquitin-like proteins which includes the Small Ubiquitin-related Modifiers (SUMO). SUMO has recently been linked to *MMP13* expression and potentially RA.[41] Thus, further work into the role of ubiquitin-like proteins in the arthritides is required.

In summary, here we describe for the first time that disruption of the UPS system is able to partially protect from OA-like cartilage destruction in the DMM murine model. Using a proteomic screen we identified TRAFD1 as a potential novel Ub target in chondrocytes and demonstrate that the depletion of the gene reduces the inflammatory induction of the potent collagenase MMP13. Further work is required to determine whether the protection from OA observed here by inhibition of the UPS is via TRAFD1, inhibition of NF-κB signalling, or perhaps via modulation of other pathways such as Wnt or TGF-β signalling.

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Running head: ubiquitination in osteoarthritis

Table 1. TUBE-enriched, Ub-associated protein with a role in NF-κB signalling

Protein #	protein accession	short name	protein description	emPAI			uniprot accession
				unst	IL-1	pIC	
1	IPI00100154	TOLLIP	Toll-interacting protein	0.1	0.1	0.1	**Q9H0E2**
2	IPI00009146	TRAFD1	TRAF-type zinc finger domain-containing protein 1	0.05	-----	-----	**O14545**
3	IPI00094740	RNF31	Isoform 1 of RING finger protein 31	-----	0.05	-----	**Q96EP0**
4	IPI00000816	YWHAE	Isoform 1 of 14-3-3 protein epsilon	-----	0.23	0.11	**P62258**
5	IPI00021263	YWHAZ	14-3-3 protein zeta/delta	0.38	-----	-----	**P63104**
6	IPI00220740	NPM1	Isoform 2 of Nucleophosmin	-----	-----	0.5	**P06748**
7	IPI00179473	SQSTM1	Isoform 1 of Sequestosome-1	0.65	0.65	0.29	**Q13501**
8	IPI00299147	SUMO3	Small ubiquitin-related modifier 3	1.1	0.64	0.64	**P55854**

* emPAI, Exponentially modified protein abundance index.[10]

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Figure legends

Figure 1. UPS inhibition prevents cartilage destruction via blockade of NF-κB signaling to MMP13. (A) and (B). Bovine nasal cartilage was cultured in serum-free medium in the presence of either medium, or medium plus IL-1 and OSM both ± MG132 or DMSO vehicle as described for 14 days. (A) Glycosaminoglycan (GAG) release into the medium was assayed using the dimethylmethylene blue assay [13] and is shown for day 7. (B). The levels of collagen fragments released into the medium were determined by measurement of hydroxyproline (OHPro).[13] Results shown are for the cumulative collagen release at day 14 of culture and expressed as a percentage of the total (mean ± SD). Experiments were performed with three bovine noses with each experiment performed in quadruplicate. (C). Treatment toxicity was measured in condition medium (day 7 and 14 - presented) using the ToxiLight™ BioAssay Kit. Data is average (n=4) emitted light from a single bovine cartilage experiment with freeze-thawed cartilage used as a positive control and set to 100% toxicity. (D). SW1353 cells or HAC were stimulated with ligands (pIC = poly(I-C)) ± MG132 or DMSO vehicle, as described and labelled, for 6 h or 24 h respectively. *MMP13* and *18S* expression were measured using real-time RT-PCR. (E). SW1353 cell were stimulated as described for 15 min and isolated protein subjected to immunoblotting with the indicated antibodies. GAPDH was used as a loading control. Real-time PCR results are combined data from 3 separate experiments (n=4/experiment) or representative of two patient HAC. Immunoblotting is representative of two experiments.

Figure 2. MG132 reduces cartilage destruction in the 'destabilisation of the medial meniscus' model *in vivo*. (A). OA in C57BL/6J mice was induced following surgical destabilization of the medial meniscus (DMM) as described. MG132 (1 mg/kg/day) or vehicle (DMSO/PEG) were delivered via subcutaneously implanted osmotic pump, with pumps replacement after 4 weeks. Data are shown at 8 weeks. Frontal sections (5 µm) were stained with haematoxylin, safranin-O/fast green. Sections shown are representative of the most affected from a selected mouse (DMSO vehicle or MG132) and show the medial compartment of the knee joint (5 x magnification). (B). Approximately fourteen sections from each mouse were graded by two blinded scorers using a validated scoring system.[16] The means of the highest score for each animal are plotted (n=8 per group) for the medial

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femoral condyle and tibial plateau and summation of both, with closed circles and open squares for vehicle or MG312 treated animals respectively. Line represents mean and error bars are SEM. Statistical significance was calculated using a Mann Whitney U test.

Figure 3. Transgenic mutated ubiquitins differentially affect DMM-induced OA. (A). OA was induced in 10-week old mice transgenic for wild-type ubiquitin, K48R or K63R mutant ubiquitin on the FVB/N genetic background. After 8 weeks animals were sacrificed and knee frontal sections (5 µm) stained with haematoxylin, safranin-O/fast green. Sections shown are representative of the most affected from a selected mouse for each genotype and show the medial compartment of the knee joint (5 x magnification). (B). Approximately fourteen sections from each mouse were graded by two blinded scorers using a validated scoring system.[16] The means of the highest score for each animal are plotted for the medial femoral condyle and tibial plateau and summation of both. C57BL/6J mice (n=22) from parallel surgery, by the same surgeon, were used to determine the effect transgenic 'wild-type' Ub. Line represents mean and error bars are SEM. Statistical significance was calculated using a Mann Whitney U test.

Figure 4. TRAFD1 is a novel ubiquitinated substrate involved in inflammatory signalling. (A). Protein extracts from SW1353 cells were enriched for poly-ubiqtuinated substrates using TUBEs as described, with colloidal coomassie staining (upper-panel) and immunoblotting with an anti-Ub (Ub; mid-panel) antibody used to verify enrichment. Enriched ubiquitin-conjugated proteins were subjected to MS. n-b is 'non-bound' protein collected after incubation with the TUBEs (B). SW1353 cells were transfected with a TRAFD1-FLAG construct or empty vector and overexpression confirmed by immunoblotting. Transfected cells were stimulated as indicated and FLAG-tagged proteins enriched by FLAG-immunoprecipitation, which was confirmed by FLAG-immunoblotting. (C). SW1353 cells were transfected with the indicated siRNA and *TRAFD1* depletion confirmed by real-time RT-PCR. (D) and (E) SW1353 cells were transfected with the indicated siRNA, stimulated with IL-1 (solid bars) or poly(I-C)(pIC) (shaded bars) as indicated for 6 or 24 h, and *MMP13* (D) or *IL6* (E) expression measured by real-time RT-PCR. Data are shown as fold change relative to the cells transfected with a non-targeting siRNA (siCon). Real-time PCR data are from 3 separate experiments performed in quadruplicate.

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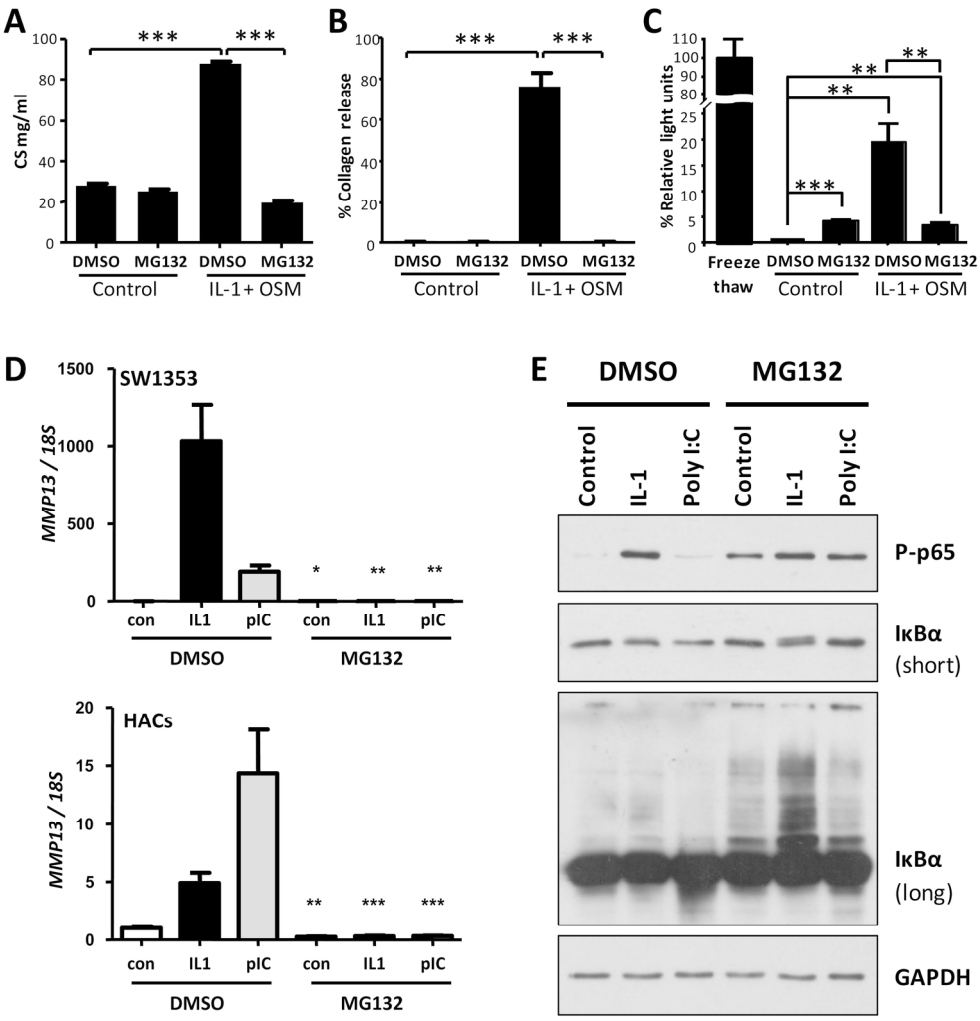

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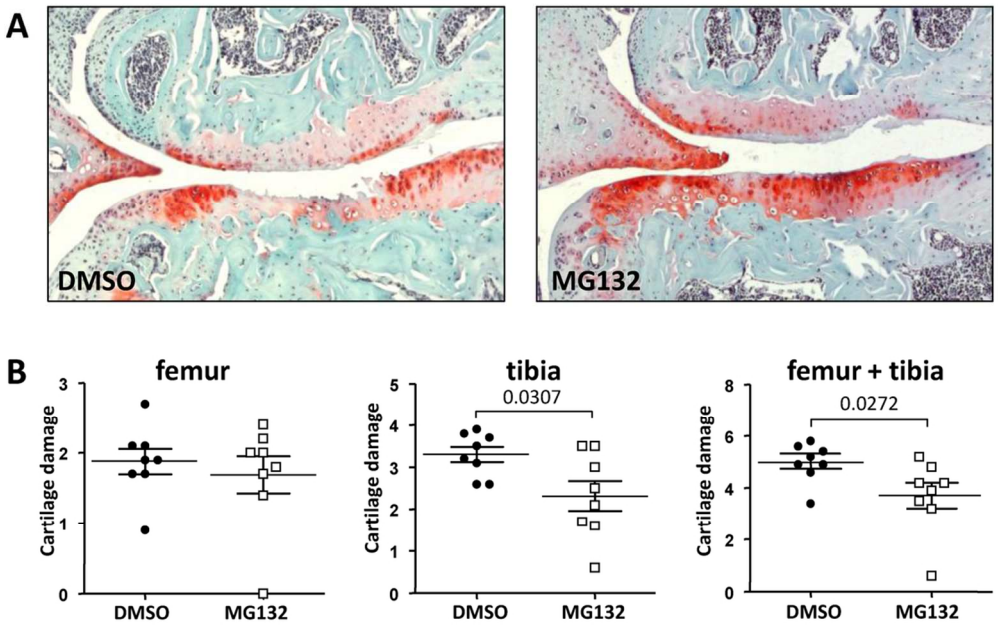

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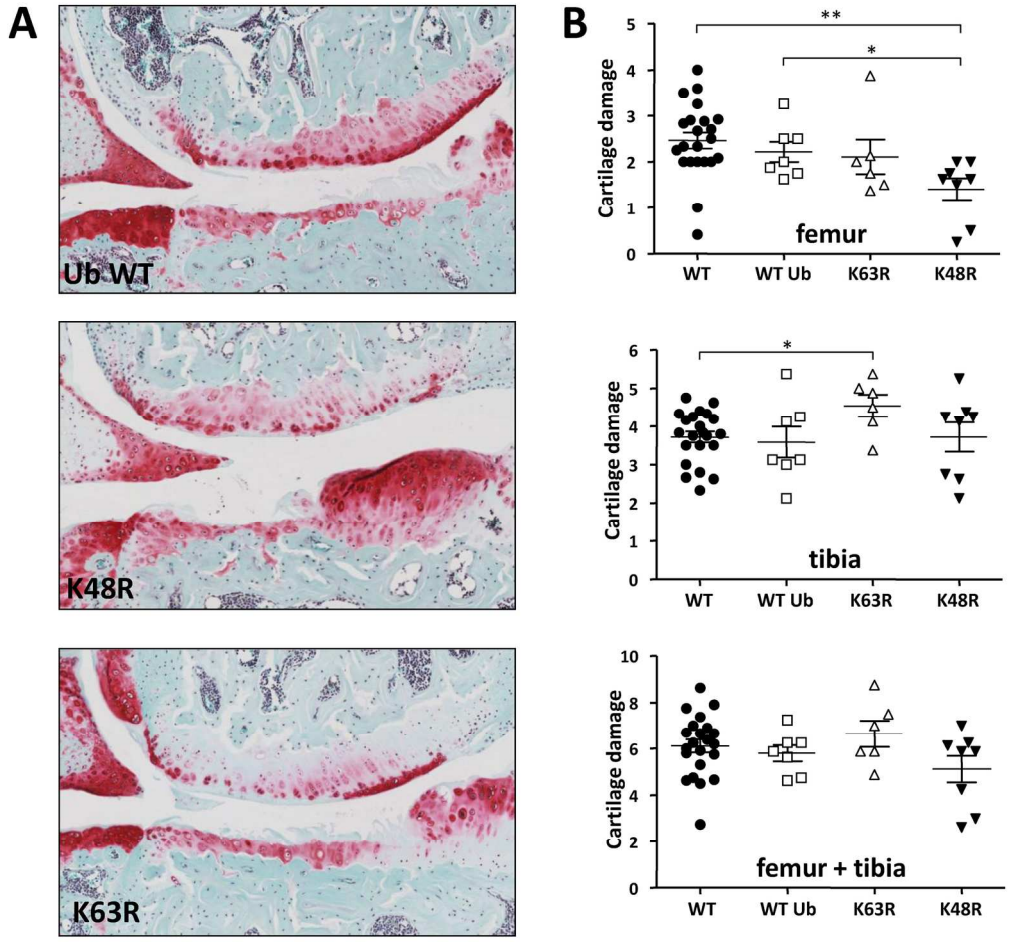
View Only



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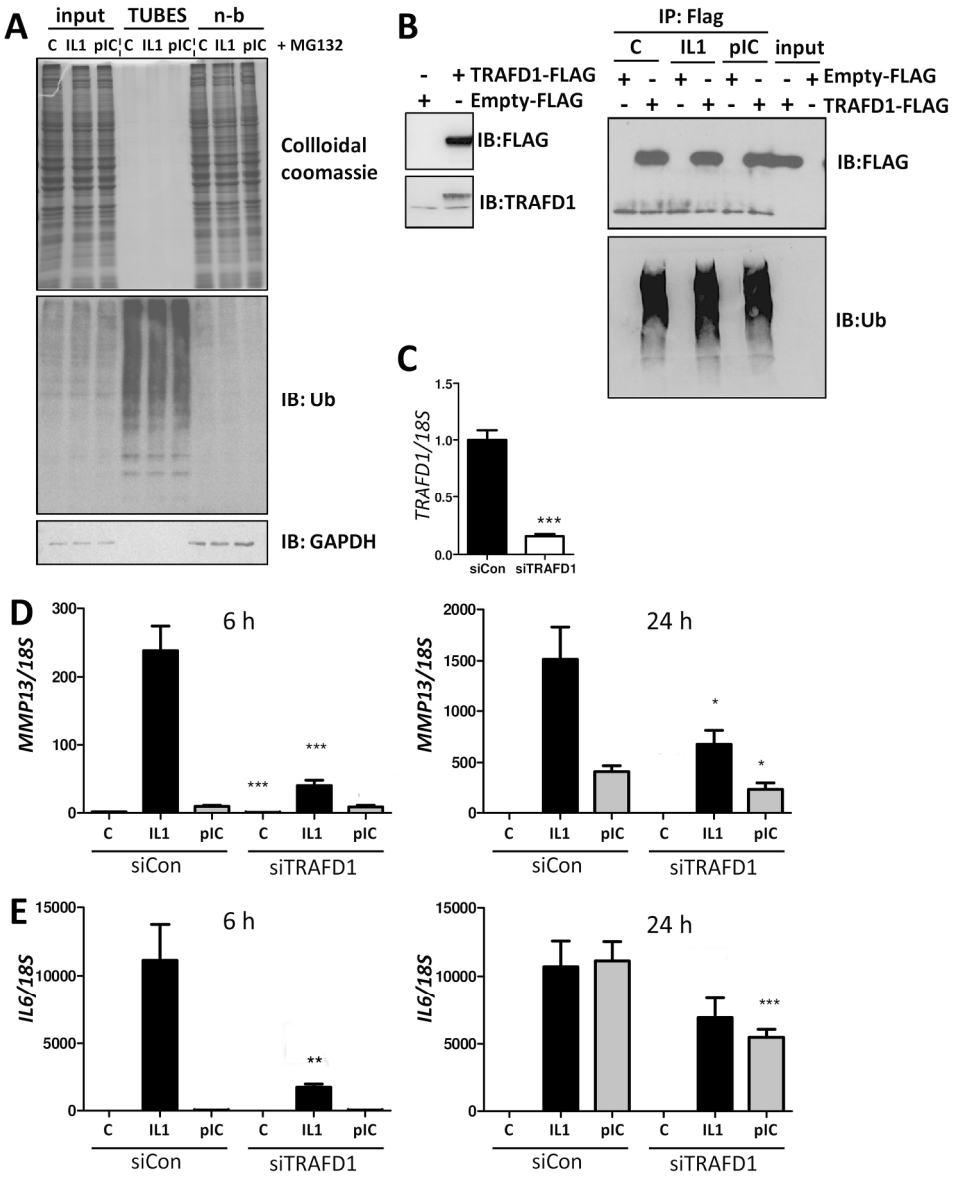

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218x265mm (300 x 300 DPI)

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Protein #	prot_acc	short name	prot_desc	emPAI unst	emPAI IL-1	emPAI pIC	prot_mass	prot_score unst	prot_score IL-1	prot_score pIC	prot_matches_sign unst	prot_matches_sign IL-1	prot_matches_sign pIC	uniprot_acc	uniprot_name
				Identified in all samples											
1	IPI00000811	PSMB6	Proteasome subunit beta type-6	0.8	1.02	1.02	25570	186	232	245	5	6	6	P28072	Proteasome subunit beta type-6
2	IPI00001639	KPNB1	Importin subunit beta-1	0.03	0.03	0.03	98420	66	71	81	1	1	1	Q14974	Importin subunit beta-1
3	IPI00001676	NPLOC4	Isoform 2 of Nuclear protein localization protein 4 homolog	0.19	0.42	0.19	70215	207	290	209	4	8	4	Q8TAT6	Nuclear protein localization protein 4 homolog
4	IPI00002134	PSMD5	26S proteasome non-ATPase regulatory subunit 5	0.06	0.06	0.06	56560	52	63	46	1	1	1	Q16401	26S proteasome non-ATPase regulatory subunit 5
5	IPI00003217	PSMB7	Proteasome subunit beta type-7	1.45	2.64	1.99	30288	378	616	470	9	13	11	Q99436	Proteasome subunit beta type-7
6	IPI00003362	HSPA5	78 kDa glucose-regulated protein	0.53	0.46	0.34	72402	312	444	330	10	9	6	P11021	78 kDa glucose-regulated protein
7	IPI00003442	HIF1A	Isoform 1 of Hypoxia-inducible factor 1-alpha	0.3	0.3	0.26	93467	285	307	259	8	8	7	Q16665	Hypoxia-inducible factor 1-alpha
8	IPI00003865	HSPA8	Isoform 1 of Heat shock cognate 71 kDa protein	2.49	2.49	2.64	71082	1206	1077	1213	30	29	30	P11142	Heat shock cognate 71 kDa protein
9	IPI00006395	GNAL	Guanine nucleotide-binding protein G(olf) subunit alpha	0.07	0.07	0.07	44794	42	50	46	1	1	1	P38405	Guanine nucleotide-binding protein G(olf) subunit alpha
10	IPI00007611	ATP5O	ATP synthase subunit O, mitochondrial	0.89	1.15	0.89	23377	241	257	177	6	7	5	P48047	ATP synthase subunit O, mitochondrial
11	IPI00007752	TUBB2C	Tubulin beta-2C chain	2.57	3.55	3.03	50255	870	995	971	25	27	25	P68371	Tubulin beta-4B chain
12	IPI00008219	RAD23A	UV excision repair protein RAD23 homolog A	0.17	0.17	0.17	39642	67	89	94	2	3	3	P54725	UV excision repair protein RAD23 homolog A
13	IPI00008433	RPS5	40S ribosomal protein S5	1.17	0.3	1.17	23033	290	101	283	6	2	6	P46782	40S ribosomal protein S5
14	IPI00008438	RPS10	40S ribosomal protein S10	0.6	0.17	0.37	18886	153	64	134	4	1	3	P46783	40S ribosomal protein S10
15	IPI00011126	PSMC1	26S protease regulatory subunit 4	2.04	2.24	2.66	49325	770	752	842	20	21	22	P62191	26S protease regulatory subunit 4
16	IPI00011253	RPS3	40S ribosomal protein S3	3.79	2.42	3.28	26842	386	366	410	14	10	12	P23396	40S ribosomal protein S3
17	IPI00011603	PSMD3	26S proteasome non-ATPase regulatory subunit 3	1.23	1.23	1.59	61054	572	532	642	16	16	19	O43242	26S proteasome non-ATPase regulatory subunit 3
18	IPI00011609	UBE3A	Isoform II of Ubiquitin-protein ligase E3A	0.13	0.03	0.06	101593	98	66	97	4	1	2	Q05086	Ubiquitin-protein ligase E3A
19	IPI00012011	CFL1	Cofilin-1	0.61	0.61	0.61	18719	191	200	176	3	3	3	P23528	Cofilin-1
20	IPI00012268	PSMD2	26S proteasome non-ATPase regulatory subunit 2	2.08	2.28	2.18	100877	1381	1580	1468	39	40	37	Q13200	26S proteasome non-ATPase regulatory subunit 2
21	IPI00013296	RPS18;RPS18P9	40S ribosomal protein S18	2.79	2.79	3.48	17708	280	277	295	7	9	9	P62269	40S ribosomal protein S18
22	IPI00014151	PSMD6	26S proteasome non-ATPase regulatory subunit 6	2.54	2.1	2.78	45787	593	602	774	20	17	20	Q15008	26S proteasome non-ATPase regulatory subunit 6
23	IPI00016832	PSMA1	Isoform Short of Proteasome subunit alpha type-1	5.8	4.03	3.54	29822	503	398	449	19	17	14	P25786	Proteasome subunit alpha type-1
24	IPI00018398	PSMC3	26S protease regulatory subunit 6A	5.33	3.12	4.6	49458	1075	820	942	30	23	30	P17980	26S protease regulatory subunit 6A
25	IPI00019927	PSMD7	26S proteasome non-ATPase regulatory subunit 7	1.09	1.09	0.77	37060	358	333	273	9	9	7	P51665	26S proteasome non-ATPase regulatory subunit 7
26	IPI00020042	PSMC4	Isoform 1 of 26S protease regulatory subunit 6B	3.67	4.31	3.67	47451	906	1033	979	25	27	25	P43686	26S protease regulatory subunit 6B
27	IPI00021290	ACLY	ATP-citrate synthase	0.03	0.03	0.03	121674	73	64	42	1	1	1	P53396	ATP-citrate synthase
28	IPI00021435	PSMC2	26S protease regulatory subunit 7	3.45	3.18	5.07	49002	1007	965	957	28	26	31	P35998	26S protease regulatory subunit 7
29	IPI00021439	ACTB	Actin, cytoplasmic 1	4.26	4.26	6.55	42052	1014	883	1400	25	22	30	P60709	Actin, cytoplasmic 1
30	IPI00021440	ACTG1	Actin, cytoplasmic 2	3.89	4.26	7.11	42108	1051	892	1400	24	22	30	P63261	Actin, cytoplasmic 2
31	IPI00022202	SLC25A3	Isoform A of Phosphate carrier protein, mitochondrial	0.16	0.16	0.08	40525	69	84	51	2	2	1	Q00325	Phosphate carrier protein, mitochondrial
32	IPI00022298	CHRNA1	Isoform 2 of Acetylcholine receptor subunit alpha	0.06	0.06	0.06	54966	44	47	45	1	1	1	P02708	Acetylcholine receptor subunit alpha
33	IPI00022694	PSMD4	Isoform Rpn10A of 26S proteasome non-ATPase regulatory subunit 4	1.26	1.82	1.43	40939	424	590	455	11	13	12	P55036	26S proteasome non-ATPase regulatory subunit 4
34	IPI00022774	VCP	Transitional endoplasmic reticulum ATPase	4.51	3.34	3.34	89950	2324	2096	2200	54	47	50	P55072	Transitional endoplasmic reticulum ATPase
35	IPI00023191	TOM1	cDNA FLJ54710, highly similar to Target of Myb protein 1	0.32	0.25	0.25	55083	287	227	184	6	4	4	B4DKQ5	cDNA FLJ54710, highly similar to Target of Myb protein 1
36	IPI00023919	PSMC5	26S protease regulatory subunit 8	4.64	3.93	4.27	45768	1124	1005	1144	28	24	27	P62195	26S protease regulatory subunit 8
37	IPI00024067	CLTC	Isoform 1 of Clathrin heavy chain 1	0.19	0.31	0.19	193260	367	598	474	11	16	11	Q00610	Clathrin heavy chain 1
38	IPI00024175	PSMA7	Isoform 1 of Proteasome subunit alpha type-7	2.62	1.92	2.25	28041	527	487	437	12	10	11	O14818	Proteasome subunit alpha type-7
39	IPI00024821	PSMD14	26S proteasome non-ATPase regulatory subunit 14	1.01	0.84	0.55	34726	283	211	199	9	7	5	O00487	26S proteasome non-ATPase regulatory subunit 14
40	IPI00025019	PSMB1	Proteasome subunit beta type-1	6.55	2.07	2.44	26700	616	487	555	17	10	11	P20618	Proteasome subunit beta type-1
41	IPI00026781	FASN	Fatty acid synthase	0.01	0.03	0.02	275877	86	146	117	1	3	2	P49327	Fatty acid synthase
42	IPI00028004	PSMB3	Proteasome subunit beta type-3	1.8	2.19	2.19	23219	456	431	430	9	9	9	P49720	Proteasome subunit beta type-3
43	IPI00028006	PSMB2	Proteasome subunit beta type-2	1.49	1.18	1.49	22993	318	301	353	8	6	7	P49721	Proteasome subunit beta type-2
44	IPI00029623	PSMA6	Proteasome subunit alpha type-6	2.65	3.53	3.07	27838	425	529	480	12	13	13	P60900	Proteasome subunit alpha type-6
45	IPI00032957	UBE2I	SUMO-conjugating enzyme UBC9	0.18	0.38	0.18	18223	64	104	80	1	2	1	P63279	SUMO-conjugating enzyme UBC9
46	IPI00033030	ADRM1	Proteasomal ubiquitin receptor ADRM1	0.24	0.33	0.15	42412	116	173	114	3	4	2	Q16186	Proteasomal ubiquitin receptor ADRM1
47	IPI00100154	TOLLIP	Toll-interacting protein	0.1	0.1	0.1	30490	56	53	52	1	1	1	Q9H0E2	Toll-interacting protein
48	IPI00105598	PSMD11	Proteasome 26S non-ATPase subunit 11 variant (Fragment)	3.32	2.35	2.57	47790	858	737	725	24	20	22	O00231	26S proteasome non-ATPase regulatory subunit 11
49	IPI00157790	KIAA0368	proteasome-associated protein ECM29 homolog	0.01	0.01	0.03	225491	58	59	85	1	1	2	Q5VYK3	Proteasome-associated protein ECM29 homolog
50	IPI00171199	PSMA3	Isoform 2 of Proteasome subunit alpha type-3	1.94	1.64	1.64	27858	339	297	293	10	9	9	P25788	Proteasome subunit alpha type-3
51	IPI00179298	HUWE1	Uncharacterized protein	0.07	0.08	0.05	485780	316	472	287	10	12	7	Q7Z6Z7	E3 ubiquitin-protein ligase HUWE1
52	IPI00179330	RPS27A	Ubiquitin-40S ribosomal protein S27a	286.43	465.95	336.9	18296	4986	5380	5210	165	173	165	P62979	Ubiquitin-40S ribosomal protein S27a
53	IPI00179473	SQSTM1	Isoform 1 of Sequestosome-1	0.65	0.65	0.29	48455	427	396	231	8	8	4	Q13501	Sequestosome-1
54	IPI00180675	TUBA1A	Tubulin alpha-1A chain	3.47	2.97	2.74	50788	1012	962	914	27	23	24	Q71U36	Tubulin alpha-1A chain
55	IPI00185374	PSMD12	26S proteasome non-ATPase regulatory subunit 12	3.43	2.52	3.43	53270	1049	771	1102	26	22	27	O00232	26S proteasome non-ATPase regulatory subunit 12
56	IPI00186290	EEF2	Elongation factor 2	0.1	0.17	0.07	96246	108	163	80	3	5	2	P13639	Elongation factor 2
57	IPI00215780	RPS19	40S ribosomal protein S19	1.5	1.5	2	16051	170	178	163	5	5	6	P39019	40S ribosomal protein S19
58	IPI00216049	HNRNPK	Isoform 1 of Heterogeneous nuclear ribonucleoprotein K	0.06	0.06	0.06	51230	52	50	59	1	1	1	P61978	Heterogeneous nuclear ribonucleoprotein K
59	IPI00217966	LDHA	Isoform 1 of L-lactate dehydrogenase A chain	0.39	0.39	0.28	36950	165	210	112	4	4	3	P00338	L-lactate dehydrogenase A chain
60	IPI00218292	UFD1L	Isoform Short of Ubiquitin fusion degradation protein 1 homolog	0.19	0.09	0.42	34763	66	53	128	2	1	4	Q92890	Ubiquitin fusion degradation protein 1 homolog
61	IPI00219018	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	0.4	0.65	0.4	36201	235	309	223	4	7	4	P04406	Glyceraldehyde-3-phosphate dehydrogenase
62	IPI00219622	PSMA2	Proteasome subunit alpha type-2	4.04	3.49	4.04	25996	621	566	630	15	13	15	P25787	Proteasome subunit alpha type-2
63	IPI00220102	DNAJB2	Isoform 3 of DnaJ homolog subfamily B member 2	0.19	0.53	0.4	35672	99	160	144	2	5	4	P25686	DnaJ homolog subfamily B member 2
64	IPI00291922	PSMA5	Proteasome subunit alpha type-5	3.36	2.89	3.36	26565	652	495	675	14	13	15	P28066	Proteasome subunit alpha type-5
65	IPI00299147	SUMO3	Small ubiquitin-related modifier 3	1.1	0.64	0.64	11687	122	133	111	4	4	3	P55854	Small ubiquitin-related modifier 3
66	IPI00299155	PSMA4	Proteasome subunit alpha type-4	3.57	5.19	4.05	29750	751	784	773	16	18	17	P25789	Proteasome subunit alpha type-4
67	IPI00299468	SCD	Acyl-CoA desaturase	0.08	0.08	0.08	41667	56	84	60	1	1	1	O00767	Acyl-CoA desaturase
68	IPI00375380	PSMD13	Isoform 2 of 26S proteasome non-ATPase regulatory subunit 13	1.87	2.3	3.07	43257	518	558	716	15	17	20	Q9UNM6	26S proteasome non-ATPase regulatory subunit 13
69	IPI00396485	EEF1A1	Elongation factor 1-alpha 1	0.62	0.94	0.72	50451	289	438	290	8	11	9	P68104	Elongation factor 1-alpha 1
70	IPI00414676	HSP90AB1	Heat shock protein HSP 90-beta	0.2	0.25	0.25	83554	203	210	185	5	6	5	P08238	Heat shock protein HSP 90-beta
71	IPI00418169	ANXA2	Isoform 2 of Annexin A2	0.45	0.57	0.45	40671	180	213	172	5	6	5	P07355	Annexin A2
72	IPI00419575	GET4	Uncharacterised protein family UPF0363 protein	0.09	0.18	0.18	36808	99	112	127	1	2	2	Q7L5D6	Golgi to ER traffic protein 4 homolog
73	IPI00440493	ATP5A1	ATP synthase subunit alpha, mitochondrial	0.05	0.11	0.17	59828	42	68	92	1	2	3	P25705	ATP synthase subunit alpha, mitochondrial
74	IPI00465128	BAG6	Isoform 1 of Large proline-rich protein BAG6	0.51	0.59	0.51	119790	540	662	552	16	18	16	P46379	Large proline-rich protein BAG6
75	IPI00465248	ENO1	Isoform alpha-enolase of Alpha-enolase	0.29	0.67	0.47	47481	193	469	275	4	8	6	P06733	Alpha-enolase
76	IPI00465439	ALDOA	Fructose-bisphosphate aldolase A	0.16	0.26	0.16	39851	82	100	85	2	3	2	P04075	Fructose-bisphosphate aldolase A
77	IPI00479186	PKM2	Isoform M2 of Pyruvate kinase isozymes M1/M2	0.69	0.78	0.78	58470	401	402	371	10	11	11	P14618	Pyruvate kinase isozymes M1/M2
78	IPI00479306	PSMB5	Isoform 1 of Proteasome subunit beta type-5	2.53	4.37	2.92	28633	536	702	615	12	16	13	P28074	Proteasome subunit beta type-5
79	IPI00479722	PSME1	Proteasome activator complex subunit 1	0.68	0.37	0.87	28876	191	114	213	5	3	5	Q06323	Proteasome activator complex subunit 1
80	IPI00555956	PSMB4	Proteasome subunit beta type-4	2.82	2.82	3.24	29242	661	700	801	13	13	14	P28070	Proteasome subunit beta type-4
81	IPI00784154	HSPD1	60 kDa heat shock protein, mitochondrial	0.11	0.11	0.33	61187	91	75	108	2	2	3	P10809	60 kDa heat shock protein, mitochondrial
82	IPI00926977	PSMC6	26S protease regulatory subunit 10B	3	1.88	2.07	46053	726	566	551	22	16	17	P62333	26S protease regulatory subunit 10B
83	IPI00930688	TUBA1B	Tubulin alpha-1B chain	4.04	3.75	3.47	50804	1198	1043	983	30	26	27	P68363	Tubulin alpha-1B chain
84	IPI00937278	PSMD8	26S proteasome non-ATPase regulatory subunit 8	0.58	0.26	0.58	39872	185	147	227	6	3	6	P48556	26S proteasome non-ATPase regulatory subunit 8
85	IPI00943181	PSME2	Uncharacterized protein	1.28	0.85	1.05	29279	342	328	338	8	5	7	Q9UL46	Proteasome activator complex subunit 2

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86	IPI00982304	PSMC3	Uncharacterized protein	3.5	2.73	5.55	15631	272	298	347	9	8	11	**E9PLQ8**	26S protease regulatory subunit 6A
			Identified in both IL-1 and poly(I:C) stimulated cells												
87	IPI00000816	YWHAE	Isoform 1 of 14-3-3 protein epsilon	-----	0.23	0.11	29326	-----	70	65	-----	2	1	**P62258**	14-3-3 protein epsilon
88	IPI00007188	SLC25A5	ADP/ATP translocase 2	-----	0.1	0.1	33059	-----	49	73	-----	1	1	**P05141**	ADP/ATP translocase 2
89	IPI00007765	HSPA9	Stress-70 protein, mitochondrial	-----	0.09	0.13	73920	-----	75	71	-----	2	2	**P38646**	Stress-70 protein, mitochondrial
90	IPI00014398	FHL1	four and a half LIM domains protein 1 isoform 5	-----	0.09	0.09	35494	-----	71	52	-----	1	1	**Q13642**	Four and a half LIM domains protein 1
91	IPI00020008	NEDD8	NEDD8	-----	0.87	0.37	9066	-----	163	136	-----	3	2	**Q15843**	NEDD8
92	IPI00218466	SEC61A1	cDNA FLJ59739, highly similar to Protein transport protein Sec61 subunit alpha isoform 1	-----	0.26	0.06	53371	-----	216	43	-----	4	1	**B4DR60**	Protein transport protein Sec61 subunit alpha isoform 0
93	IPI00031691	RPL9	60S ribosomal protein L9	-----	0.15	0.31	21964	-----	49	101	-----	1	2	**P32969**	60S ribosomal protein L9
94	IPI00293464	DDB1	DNA damage-binding protein 1	-----	0.05	0.02	128142	-----	71	41	-----	2	1	**Q16531**	DNA damage-binding protein 1
95	IPI00302592	FLNA	Isoform 2 of Filamin-A	-----	0.09	0.1	282581	-----	292	285	-----	8	9	**Q60FE6**	Filamin A
96	IPI00328354	MAGED1	Isoform 1 of Melanoma-associated antigen D1	-----	0.04	0.04	86221	-----	50	80	-----	1	1	**Q9Y5V3**	Melanoma-associated antigen D1
97	IPI00446294	TOM1L2	Isoform 1 of TOM1-like protein 2	-----	0.06	0.06	55864	-----	47	66	-----	1	1	**Q6ZVM7**	TOM1-like protein 2
98	IPI00790498	PSMD11	PSMD11 Protein	-----	2.14	2.7	18111	-----	261	289	-----	8	9	**-**	-
99	IPI01019113	TUBB	Tubulin beta chain	-----	3.31	3.31	50095	-----	1010	1067	-----	26	26	**P07437**	Tubulin beta chain
			Identified only in unstimulated cells												
100	IPI00000643	BAG2	BAG family molecular chaperone regulator 2	0.13	-----	-----	23928	46	-----	-----	1	-----	-----	**O95816**	BAG family molecular chaperone regulator 2
101	IPI00008455	MYO6	Isoform 2 of Myosin-VI	0.02	-----	-----	147324	49	-----	-----	1	-----	-----	**Q9UM54**	Unconventional myosin-VI
102	IPI00009146	TRAFD1	TRAF-type zinc finger domain-containing protein 1	0.05	-----	-----	66226	55	-----	-----	1	-----	-----	**O14545**	TRAF-type zinc finger domain-containing protein 1
103	IPI00019472	SLC1A5	Neutral amino acid transporter B(0)	0.05	-----	-----	57018	53	-----	-----	1	-----	-----	**Q15758**	Neutral amino acid transporter B(0)
104	IPI00021263	YWHAZ	14-3-3 protein zeta/delta	0.38	-----	-----	27899	100	-----	-----	3	-----	-----	**P63104**	14-3-3 protein zeta/delta
105	IPI00022891	SLC25A4	ADP/ATP translocase 1	0.2	-----	-----	33271	89	-----	-----	2	-----	-----	**P12235**	ADP/ATP translocase 1
106	IPI00027255	MYL6B	Myosin light chain 6B	0.14	-----	-----	22864	49	-----	-----	1	-----	-----	**P14649**	Myosin light chain 6B
107	IPI00027626	CCT6A	T-complex protein 1 subunit zeta	0.11	-----	-----	58444	56	-----	-----	1	-----	-----	**P40227**	T-complex protein 1 subunit zeta
108	IPI00028635	RPN2	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	0.05	-----	-----	69355	49	-----	-----	1	-----	-----	**P04844**	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase sub
109	IPI00030243	PSME3	Isoform 1 of Proteasome activator complex subunit 3	0.11	-----	-----	29602	51	-----	-----	1	-----	-----	**P61289**	Proteasome activator complex subunit 3
110	IPI00030770	PSMG1	Isoform 1 of Proteasome assembly chaperone 1	0.09	-----	-----	33631	44	-----	-----	1	-----	-----	**O95456**	Proteasome assembly chaperone 1
111	IPI00075248	CALM3;CALM2;CAI	Calmodulin	0.19	-----	-----	16827	40	-----	-----	0	-----	-----	**P62158**	Calmodulin
112	IPI00149276	BRE	Isoform 1 of BRCA1-A complex subunit BRE	0.07	-----	-----	47572	48	-----	-----	1	-----	-----	**Q9NXR7**	BRCA1-A complex subunit BRE
113	IPI00218343	TUBA1C	Tubulin alpha-1C chain	3	-----	-----	50548	1045	-----	-----	26	-----	-----	**Q9BQE3**	Tubulin alpha-1C chain
114	IPI00294501	DHCR7	7-dehydrocholesterol reductase	0.06	-----	-----	55195	43	-----	-----	1	-----	-----	**Q9UBM7**	7-dehydrocholesterol reductase
115	IPI00295772	CYP51A1	lanosterol 14-alpha demethylase isoform 1	0.05	-----	-----	57641	60	-----	-----	1	-----	-----	**Q16850**	Lanosterol 14-alpha demethylase
116	IPI00553169	FLNA	Uncharacterized protein	0.08	-----	-----	248165	189	-----	-----	5	-----	-----	**A6NDY9**	Filamin A
117	IPI00645452	TUBB	Uncharacterized protein	3.02	-----	-----	48135	980	-----	-----	27	-----	-----	**B7ZAK1**	cDNA, FLJ79215, highly similar to Tubulin beta-7 chain
118	IPI00966795	HSPA9	Uncharacterized protein	0.5	-----	-----	6805	53	-----	-----	1	-----	-----	**D6RA73**	Stress-70 protein, mitochondrial
			Identified only in IL-1 stimulated cells												
119	IPI00000875	EEF1G	highly similar to Elongation factor 1-gamma	-----	0.06	-----	56456	-----	40	-----	-----	1	-----	**B4DTG2**	Elongation factor 1-gamma
120	IPI00013808	ACTN4	Alpha-actinin-4	-----	0.03	-----	105245	-----	41	-----	-----	1	-----	**O43707**	Alpha-actinin-4
121	IPI00014311	CUL2	Cullin-2	-----	0.07	-----	87554	-----	60	-----	-----	2	-----	**Q13617**	Cullin-2
122	IPI00015671	TUBAL3	Isoform 1 of Tubulin alpha chain-like 3	-----	0.13	-----	50675	-----	115	-----	-----	2	-----	**A6NHL2**	Tubulin alpha chain-like 3
123	IPI00022744	CSE1L	Isoform 1 of Exportin-2	-----	0.03	-----	111145	-----	41	-----	-----	1	-----	**P55060**	Exportin-2
124	IPI00094740	RNF31	Isoform 1 of RING finger protein 31	-----	0.05	-----	122654	-----	103	-----	-----	2	-----	**Q96EP0**	E3 ubiquitin-protein ligase RNF31
125	IPI00215948	CTNNA1	Isoform 1 of Catenin alpha-1	-----	0.03	-----	100693	-----	81	-----	-----	1	-----	**P35221**	Catenin alpha-1
126	IPI00216691	PFN1	Profilin-1	-----	0.21	-----	15216	-----	51	-----	-----	1	-----	**P07737**	Profilin-1
127	IPI00217407	UBR2	Isoform 4 of E3 ubiquitin-protein ligase UBR2	-----	0.03	-----	203831	-----	72	-----	-----	2	-----	**Q8IWV8**	E3 ubiquitin-protein ligase UBR2
128	IPI00221092	RPS16	40S ribosomal protein S16	-----	0.43	-----	16549	-----	86	-----	-----	2	-----	**P62249**	40S ribosomal protein S16
129	IPI00410680	ANKHD1-EIF4EBP3	Isoform 3 of Ankyrin repeat and KH domain-containing protein 1	-----	0.05	-----	64585	-----	41	-----	-----	1	-----	**Q8IWZ3**	Ankyrin repeat and KH domain-containing protein 1
130	IPI00423874	AMFR	E3 ubiquitin-protein ligase AMFR	-----	0.04	-----	73747	-----	39	-----	-----	1	-----	**Q9UKV5**	E3 ubiquitin-protein ligase AMFR
131	IPI00456695	PSMD1	Isoform 2 of 26S proteasome non-ATPase regulatory subunit 1	-----	2.82	-----	103162	-----	2134	-----	-----	46	-----	**Q99460**	26S proteasome non-ATPase regulatory subunit 1
132	IPI00643152	HSPA1L	highly similar to Heat shock 70 kDa protein 1L	-----	0.22	-----	77912	-----	214	-----	-----	5	-----	**B4DI54**	cDNA FLJ56386, highly similar to Heat shock 70 kDa protein 1L
133	IPI00759683	CAV1	Isoform Beta of Caveolin-1	-----	0.19	-----	17183	-----	43	-----	-----	1	-----	**Q03135**	Caveolin-1
134	IPI01025580		- Possible J 56 gene segment (Fragment)	-----	1.75	-----	2269	-----	41	-----	-----	1	-----	**A0N4V7**	HCG2039797
			Identified only in poly(I:C) stimulated cells												
135	IPI00008524	PABPC1	Isoform 1 of Polyadenylate-binding protein 1	-----	-----	0.14	70854	-----	-----	130	-----	-----	3	**P11940**	Polyadenylate-binding protein 1
136	IPI00008527	RPLP1	60S acidic ribosomal protein P1	-----	-----	0.64	11621	-----	-----	80	-----	-----	2	**P05386**	60S acidic ribosomal protein P1
137	IPI00008529	RPLP2	60S acidic ribosomal protein P2	-----	-----	0.64	11658	-----	-----	99	-----	-----	2	**P05387**	60S acidic ribosomal protein P2
138	IPI00008530	RPLP0	60S acidic ribosomal protein P0	-----	-----	0.42	34423	-----	-----	123	-----	-----	4	**P05388**	60S acidic ribosomal protein P0
139	IPI00012442	G3BP1	Ras GTPase-activating protein-binding protein 1	-----	-----	0.06	52189	-----	-----	69	-----	-----	1	**Q13283**	Ras GTPase-activating protein-binding protein 1
140	IPI00013415	RPS7	40S ribosomal protein S7	-----	-----	0.31	22113	-----	-----	61	-----	-----	2	**P62081**	40S ribosomal protein S7
141	IPI00013683	TUBB3;MC1R	Tubulin beta-3 chain	-----	-----	1.05	50856	-----	-----	419	-----	-----	14	**Q13509**	Tubulin beta-3 chain
142	IPI00013917	RPS12	40S ribosomal protein S12	-----	-----	0.22	14905	-----	-----	62	-----	-----	1	**P25398**	40S ribosomal protein S12
143	IPI00021405	LMNA	Isoform A of Prelamin-A/C	-----	-----	0.39	74380	-----	-----	188	-----	-----	7	**P02545**	Prelamin-A/C
144	IPI00021428	ACTA1	Actin, alpha skeletal muscle	-----	-----	1.54	42366	-----	-----	409	-----	-----	13	**P68133**	Actin, alpha skeletal muscle
145	IPI00024933	RPL12	Isoform 1 of 60S ribosomal protein L12	-----	-----	0.64	17979	-----	-----	95	-----	-----	3	**P30050**	60S ribosomal protein L12
146	IPI00026087	BANF1	Barrier-to-autointegration factor	-----	-----	0.32	10280	-----	-----	46	-----	-----	1	**O75531**	Barrier-to-autointegration factor
147	IPI00026302	RPL31	60S ribosomal protein L31	-----	-----	0.5	14454	-----	-----	90	-----	-----	2	**P62899**	60S ribosomal protein L31
148	IPI00027252	PHB2	Prohibitin-2	-----	-----	0.09	33276	-----	-----	44	-----	-----	1	**Q99623**	Prohibitin-2
149	IPI00031562	HIST3H2A	Histone H2A type 3	-----	-----	0.86	14113	-----	-----	138	-----	-----	3	**Q7L7L0**	Histone H2A type 3
150	IPI00031801	CSDA	Isoform 1 of DNA-binding protein A	-----	-----	0.08	40066	-----	-----	86	-----	-----	1	**P16989**	DNA-binding protein A
151	IPI00072918	COL6A3	Uncharacterized protein	-----	-----	0.02	323698	-----	-----	89	-----	-----	2	**E7ENL6**	Collagen alpha-3(VI) chain
152	IPI00178352	FLNC	Isoform 1 of Filamin-C	-----	-----	0.01	293407	-----	-----	51	-----	-----	1	**Q14315**	Filamin-C
153	IPI00215965	HNRNPA1	Isoform A1-B of Heterogeneous nuclear ribonucleoprotein A1	-----	-----	0.08	38837	-----	-----	58	-----	-----	1	**P09651**	Heterogeneous nuclear ribonucleoprotein A1
154	IPI00219153	RPL22	60S ribosomal protein L22	-----	-----	0.22	14835	-----	-----	51	-----	-----	1	**P35268**	60S ribosomal protein L22
155	IPI00220278	MYL9	Myosin regulatory light polypeptide 9	-----	-----	3.46	19871	-----	-----	388	-----	-----	10	**P24844**	Myosin regulatory light polypeptide 9
156	IPI00220403	HIST1H2BB	Histone H2B type 1-B	-----	-----	0.87	13942	-----	-----	155	-----	-----	3	**P33778**	Histone H2B type 1-B
157	IPI00220740	NPM1	Isoform 2 of Nucleophosmin	-----	-----	0.5	29617	-----	-----	147	-----	-----	4	**P06748**	Nucleophosmin
158	IPI00335168	MYL6	Isoform Non-muscle of Myosin light polypeptide 6	-----	-----	4.63	17090	-----	-----	326	-----	-----	10	**P60660**	Myosin light polypeptide 6
159	IPI00419880	SNORD73A;RPS3A	40S ribosomal protein S3a	-----	-----	0.1	30154	-----	-----	65	-----	-----	1	**P61247**	40S ribosomal protein S3a
160	IPI00444262	NCL	highly similar to Nucleolin	-----	-----	0.52	65979	-----	-----	412	-----	-----	9	**Q6ZS99**	cDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nucle
			Idfentified in unstimulated and IL-1 stimulated cells												
161	IPI00005705	PPP1CC	Isoform Gamma-1 of Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	0.08	0.08	-----	37701	57	82	-----	1	1	-----	**P36873**	Serine/threonine-protein phosphatase PP1-gamma catalytic subuni
162	IPI00006482	ATP1A1	Isoform Long of Sodium/potassium-transporting ATPase subunit alpha-1	0.03	0.06	-----	114135	96	63	-----	1	2	-----	**P05023**	Sodium/potassium-transporting ATPase subunit alpha-1
163	IPI00008603	ACTA2	Actin, aortic smooth muscle	1.05	1.2	-----	42381	279	284	-----	10	10	-----	**P62736**	Actin, aortic smooth muscle
164	IPI00022200	COL6A3	Isoform 1 of Collagen alpha-3(VI) chain	0.09	0.01	-----	344167	152	175	-----	4	4	-----	**P12111**	Collagen alpha-3(VI) chain
165	IPI00182289	RPS29	40S ribosomal protein S29	0.49	0.49	-----	6900	56	64	-----	1	1	-----	**P62273**	40S ribosomal protein S29
166	IPI00217831	ANKRD13A	Ankyrin repeat domain-containing protein 13A	0.09	0.05	-----	67976	86	47	-----	2	1	-----	**Q8IZ07**	Ankyrin repeat domain-containing protein 13A
167	IPI00791265	SNORD54;RPS20	Uncharacterized protein	0.49	1.23	-----	6881	50	89	-----	1	2	-----	**E5RJX2**	40S ribosomal protein S20
			Identified in unstimulated and poly(I:C) stimulated cells												
168	IPI00019502	MYH9	Isoform 1 of Myosin-9	0.1	-----	1.51	227646	340	-----	2994	7	-----	66	**P35579**	Myosin-9

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169	IPI00026271	RPS14	40S ribosomal protein S14	0.2	-----	0.43	16434	59	-----	93	1	-----	2	**P62263**	40S ribosomal protein S14
170	IPI00033494	MYL12B	Myosin regulatory light chain 12B	0.16	-----	5.99	19824	66	-----	584	1	-----	13	**O14950**	Myosin regulatory light chain 12B
171	IPI00299608	PSMD1	Isoform 1 of 26S proteasome non-ATPase regulatory subunit 1	2.45	-----	2.35	106795	1808	-----	1909	45	-----	43	**Q99460**	26S proteasome non-ATPase regulatory subunit 1
172	IPI00303476	ATP5B	ATP synthase subunit beta, mitochondrial	0.06	-----	0.11	56525	49	-----	89	1	-----	2	**P06576**	ATP synthase subunit beta, mitochondrial
173	IPI00418471	VIM	Vimentin	0.25	-----	2.11	53676	100	-----	621	4	-----	20	**P08670**	Vimentin
174	IPI00450975	RPS16	Uncharacterized protein	0.67	-----	0.67	17267	102	-----	120	3	-----	3	**Q6IPX4**	40S ribosomal protein S16
175	IPI00797373	DOCK8	Isoform 1 of Dedicator of cytokinesis protein 8	0.01	-----	0.01	240886	53	-----	42	1	-----	1	**Q8NF50**	Dedicator of cytokinesis protein 8
176	IPI00968128	RPL9	Protein	0.15	-----	0.32	21671	45	-----	81	1	-----	2	-	-

Modified peptides Ubiquitin ?	K #
MQIFVK(GG)TLTGK	
LIFAGK(GG)QLEDGR	
TLSDYNIQK(GG)ESTLHLVLR	
TLTGK(GG)TITLEVEPSDTIENVK	
LIFAGK(GG)QLEDGRTLSDYNIQK	

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